The EMBO Journal vol.12 no.2 pp.625-630, 1993

Regulation of the *Escherichia coli rmf* gene encoding the ribosome modulation factor: growth phase- and growth rate-dependent control

Masahiro Yamagishi, Hiroshi Matsushima, Akira Wada[1], Masayuki Sakagami, Nobuyuki Fujita and Akira Ishihama

Department of Molecular Genetics, National Institute of Genetics, Mishima, Shizuoka 411 and [1]Department of Physics, Faculty of Science, Kyoto University, Sakyo-ku, Kyoto 606, Japan

Communicated by A.A.Travers

Ribosome modulation factor (RMF) is a protein specifically associated with 100S ribosome dimers which start to accumulate in *Escherichia coli* cells upon growth transition from exponential to stationary phase. The structural gene, *rmf*, encoding the 55 amino acid residues RMF protein has been cloned from the 21.8 min region of the *E.coli* genome and sequenced. While *rmf* was silent in rapidly growing exponential phase cells, a high level of transcription took place concomitantly with the growth transition to stationary phase. Under slow growth conditions, *rmf* was expressed even in exponential phase and there was an inverse relationship between the expression of *rmf* and the cell growth rate. Thus, the expression profile of *rmf* is contrary to those of genes for ribosomal components and ribosome-associated proteins constituting the translational apparatus. The *katF* gene product, a stationary phase-specific σ factor, was not required for the expression of *rmf*. Disruption of *rmf* resulted in loss of ribosome dimers and reduction of cell viability during stationary phase.

Key words: Escherichia coli/growth rate-dependent control/ ribosome dimer/ribosome modulation factor/stationary phase

Introduction

As unicellular organisms, bacteria have a wide range of adaptability to changes in environment. While bacterial cells can divide rapidly under appropriate growth conditions, they enter into stationary phase to cease growth when exposed to unfavorable circumstances and yet retain viability for a considerable period. Adaptation to non-growing state requires many physiological changes including reduction of global gene expression, accompanied by induction of stationary phase-specific genes (for a review see Siegele and Kolter, 1992). However, the gene regulation mechanisms involved in the adaptation to stationary phase are largely unknown.

We have focused on possible alterations in function and structure of RNA polymerase and ribosomes in stationary phase *Escherichia coli* cells. RNA polymerase and ribosomes constitute a central core apparatus for gene expression, and mechanisms to maintain them at appropriate levels during stationary phase without irreversible loss of their functions are essential not only for the expression of required genes in the non-growing state but also for efficient resumption of growth when cells are exposed to favorable circumstances. Recently, we identified stationary phase-specific forms of RNA polymerase (Ozaki *et al.*, 1991, 1992).

In parallel, we characterized 100S ribosomes which are dimers of 70S ribosomes seen in extracts prepared from stationary phase cells (McCarthy, 1960; Feiss and DeMoss, 1965; Wada *et al.*, 1990). Although the functional role of ribosome dimerization is not yet known, dimers are rapidly converted to 70S ribosomes upon transfer of the stationary phase cells into fresh medium (McCarthy, 1960; Wada *et al.*, 1990), indicating that the dimeric ribosomes are recycled at the onset of the next round of exponential growth. Analysis of the protein composition of ribosome dimers permitted us to identify RMF (ribosome modulation factor), a novel protein associated with ribosome dimers (Wada *et al.*, 1990).

To approach an understanding of the role of RMF, we analyzed the structure, function and synthesis of RMF. In this report, we describe the cloning and sequencing of the *rmf* gene encoding RMF and its regulation under various growth conditions. The results indicate that the expression of *rmf* depends not only on growth phase but also, inversely, on growth rate. This is the first example of an inverse relationship between gene expression and growth rate among genes encoding ribosomal components and ribosome-associated proteins. When the *rmf* gene on the chromosome was disrupted, the resultant mutant lost viability during stationary phase and ribosome dimers were not observed in extracts prepared from its stationary phase cells.

Results

Cloning and sequence determination of rmf

Previously, we determined the partial amino acid sequence of RMF isolated from *E.coli* strain W3110 and, after searching for the deduced DNA sequence in DNA data banks, predicted that *rmf* is located downstream of *fabA* but transcribed in a convergent direction (Wada *et al.*, 1990). To prove this prediction, we cloned the corresponding chromosomal region into plasmids from a lambda clone (λ222) in Kohara's phage library of *E.coli* W3350 chromosomal DNA (Kohara *et al.*, 1987) and determined the nucleotide sequence. As shown in Figure 1, the deduced amino acid sequence contains the partial amino acid sequence of RMF determined previously (see Wada *et al.*, 1990) except that the third residue from the N-terminus is arginine instead of isoleucine. To confirm this result, the region between primers 1 and 2 (see Figure 1) was amplified directly from the chromosomal DNA of W3110 by PCR and sequenced. No difference was found between the two DNA sequences. This discrepancy from the protein sequence is probably due to misreading of a weak arginine signal, which is sometimes difficult to distinguish from isoleucine in amino acid sequence analysis. Thus, we concluded that *rmf* is located downstream of *fabA* as previously predicted, and that

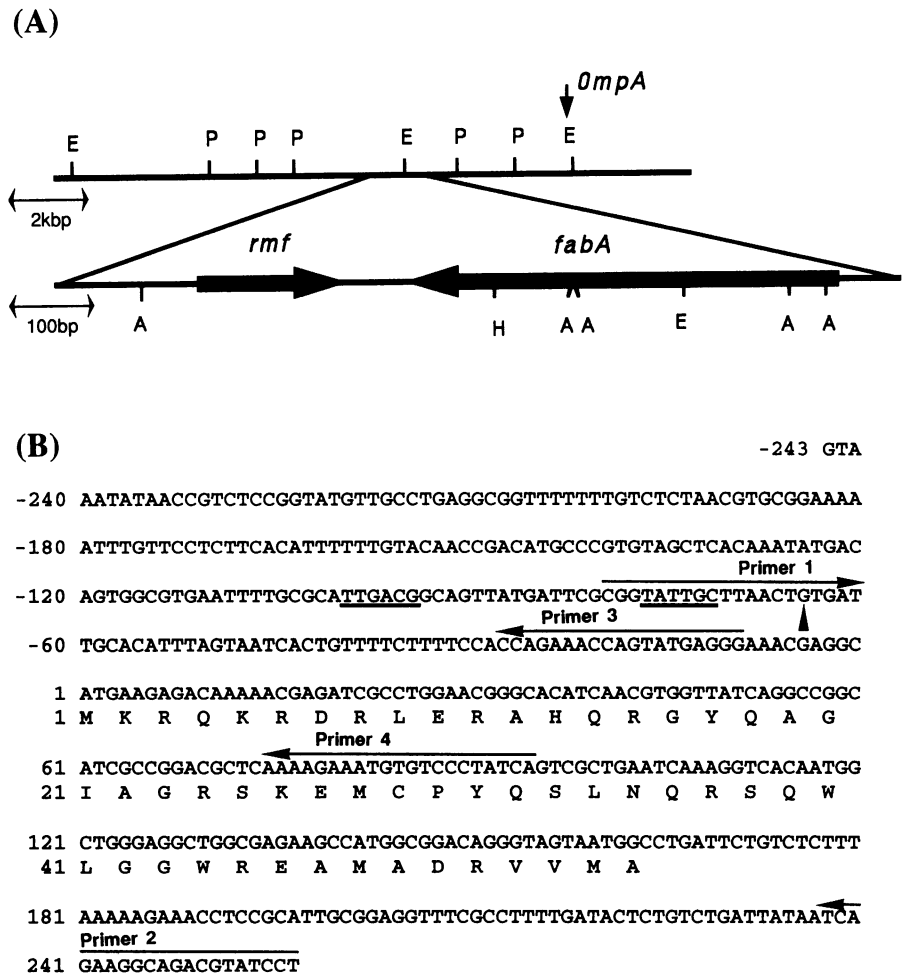

Fig. 1. (**A**) Restriction enzyme map of a chromosomal region containing *rmf*. The coding regions of *rmf* and *fabA* and the directions of transcription are shown by arrowed black boxes. The location of *ompA* is shown by a vertical arrow. Restriction enzyme sites are A, *Acc*II; E, *Eco*RV; H, *Hin*cII; P, *Pst*I. (**B**) Nucleotide and deduced amino acid sequences of *rmf*. The positions and directions of primers are shown by arrows. The transcription start site is indicated by an arrowhead and the −35 and −10 signals of the predicted promoter are underlined.

RMF is composed of 55 amino acid residues with a molecular mass of 6480. RMF is a basic protein (pI = 11.7) and is rich in arginine and lysine. No other protein with a significant similarity to RMF was found in the current sequence data banks.

Growth phase-dependent expression of rmf

To determine whether the expression of *rmf* is dependent on growth phase, we examined the level of mRNA by Northern hybridization (Figure 2B). *rmf* mRNA was not detectable in rapidly growing exponential phase cells of W3110 in nutrient-rich LB medium. During the transition from exponential to stationary phase, however, *rmf* mRNA began to accumulate. Thus, the transcription of *rmf* is clearly regulated in a growth phase-dependent manner.

In order to obtain quantitative data on the expression of *rmf* by a convenient assay, a lambda phage carrying a protein fusion between *rmf* and *lacZ*, *rmf*−*lacZ*, was lysogenized into the W3110 chromosome to construct strain HMY13 (see Materials and methods). The β-galactosidase activity began to increase concomitantly with growth transition from exponential to stationary phase (Figure 2A), roughly in parallel to the increase of *rmf* mRNA (Figure 2B). The rapid increase and the high plateau level of β-galactosidase activity suggest that the *rmf* promoter is very active during the growth transition to stationary phase, while it is almost silent in rapidly growing cells. The *katF* (*rpoS*) product, a possible principal σ factor (σ^S) during stationary phase, is required for the expression of some stationary phase-specific genes (see Discussion). However, *rmf*−*lacZ* was induced in the normal way upon growth transition into stationary phase in strain HMY14 carrying *katF*::Tn*10* (Figure 2A).

The transcriptional start site determined by primer extension (Figure 3) was located 65 bases upstream of the initiation codon as indicated in Figure 1. As expected, the primer extension product was detected in stationary phase cells but not in exponential phase cells. The position of the start site was confirmed by S1 nuclease protection (data not shown). The −35 and −10 regions of a possible promoter are underlined in Figure 1 (see Discussion).

Growth rate-dependent expression of rmf

Since decline of growth rate during growth phase transition is followed by the induction of *rmf*, it is possible that the expression of this gene in exponential phase cells is also under growth rate-dependent control. HMY13 carrying *rmf*−*lacZ* as described above was grown in various kinds of media and β-galactosidase activity was measured during exponential phase. As shown in Figure 4, the expression of *rmf* was observed in exponentially growing cells at low

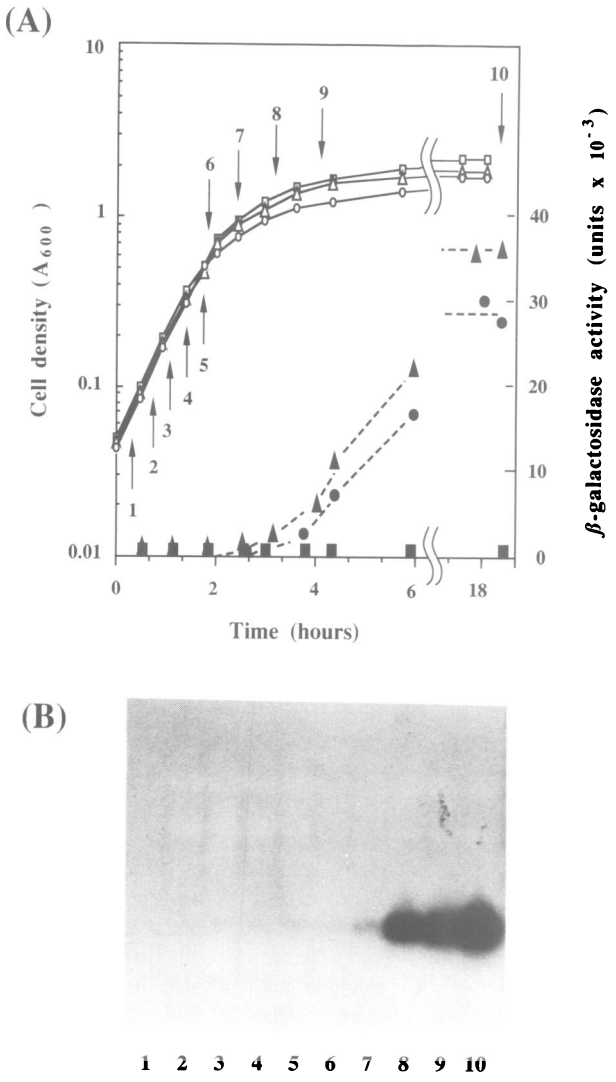

Fig. 2. (**A**) Cell growth curve and growth phase-dependent induction of *rmf–lacZ*. Strains W3110 (open squares), HMY13 (open circles) and HMY14 (open triangles) were grown in LB medium at 37°C and at the times indicated, β-galactosidase activity was determined (filled squares, W3110; filled circles, HMY13; filled triangles, HMY14). (**B**) Growth phase-dependent induction of *rmf* as detected by Northern hybridization. Portions of the culture of W3110 were taken at the times indicated by arrows in (A) and used for Northern hybridization. Ten micrograms of total RNA prepared from each sample were separated by electrophoresis, blotted onto a nitrocellulose filter and hybridized with a ^{32}P-labeled RNA probe (see Materials and methods).

growth rates and the expression level was inversely dependent on growth rate. Essentially the same expression pattern was obtained for *rmf–lacZ* when strain CSH26 containing the *lac–pro* deletion was used as a host instead of W3110 or when the fusion gene was on a plasmid (data not shown). No significant effect of the disruption of *katF* was observed on the growth rate-dependent expression of *rmf–lacZ* (strain HMY13, Figure 4).

Viability and 100S ribosome formation during stationary phase

The *rmf* gene on the chromosome of strain W3110 was disrupted by insertion of the chloramphenicol resistance gene (see Materials and methods). The resultant *rmf*::Cm^r strain, HMY15, grew at the normal rate during exponential phase in nutrient-rich media (LB or EP + glucose) at 37°C. After

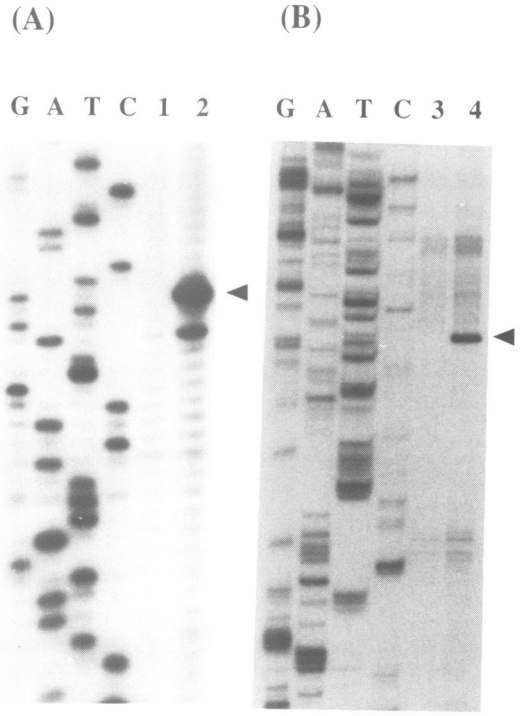

Fig. 3. Mapping of the transcription start site of *rmf*. One picomole each of ^{32}P-labeled primer [(**A**) primer 3; (**B**) primer 4 (see Figure 1)] was hybridized to 10 μg of total RNA prepared from exponential phase cells (lanes 1 and 3) or stationary phase cells (lanes 2 and 4) of W3110 grown in LB medium at 37°C, and elongated by MMV reverse transcriptase. Products were analyzed, together with reference sequence ladders, on a 7% polyacrylamide gel.

entering into stationary phase, however, the mutant strain lacking *rmf* lost viability much more rapidly than the parent. The results shown in Figure 5 are from an example in which EP + glucose medium was used for cell culture. The difference in rate of viability loss between the *rmf*::Cm^r strain and the parent in stationary phase was more drastic in LB medium than in EP + glucose medium, but even the parent lost viability substantially during the first several days in this medium (data not shown). These results show that *rmf* is required for prolonged survival during stationary phase.

In order to examine whether ribosome dimers were formed in the *rmf*::Cm^r strain, portions of the culture described above were collected and cell extracts were analyzed by sucrose gradient centrifugation (Figure 6). During exponential phase, a major peak of 70S ribosomes and small amounts of 50S and 30S subunits were observed regardless of whether the strain carried intact or disrupted *rmf*. After reaching stationary phase, ~60% of ribosomes in extracts from the parent strain sedimented as dimers in a sucrose gradient (Figure 6C). In contrast, dimers were not detected in extracts prepared from stationary phase cells of the *rmf*::Cm^r strain (Figure 6D). Samples were also taken at several times other than those shown in Figure 5, but there was no indication of ribosome dimer formation. Therefore, *rmf* is essential for ribosome dimer formation in stationary phase cells.

Discussion

RMF was identified as a small protein associated with ribosome dimers in stationary phase *E.coli* cells (Wada

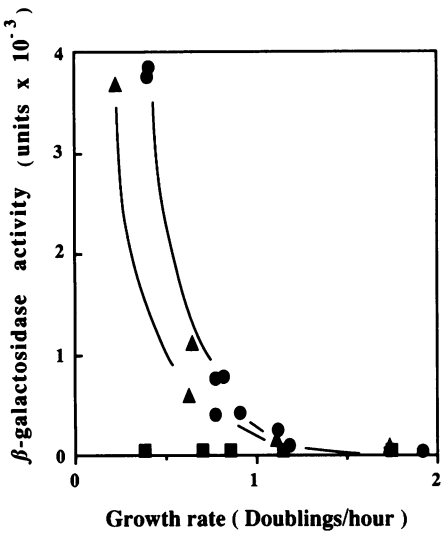

Fig. 4. Growth rate-dependent expression of *rmf–lacZ*. Strains W3110 (filled squares) and HMY13 (filled circles) and HMY14 (filled triangles) were grown in LB, M9A + glucose, M9 + glucose, M9A + glycerol, or M9 + glycerol media at 37°C. Exponential phase cells were assayed for β-galactosidase activity.

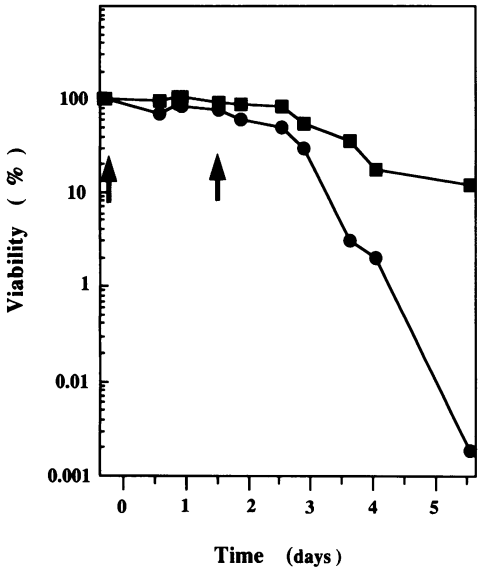

Fig. 5. Cell viability during stationary phase. Strains W3110 (filled squares) and HMY15 (filled circles) were grown in EP + glucose medium at 37°C and viability was determined by counting colony numbers in aliquots of the culture grown on LB plates at 37°C. Cell growth virtually ceased at time 0. At the times indicated by arrows, cells were collected for sedimentation analysis of ribosomes (Figure 6).

et al., 1990). The protein is undetectable in ribosome fractions (Wada *et al.*, 1990) and in total cell extracts (Wada,A., unpublished) prepared from rapidly growing cells. In this study, we have cloned the gene, *rmf*, encoding RMF and confirmed its predicted chromosomal location (21.8 min) (Wada *et al.*, 1990). The deduced amino acid sequence of 55 amino acid residues has no significant similarity to any protein in the current sequence data bases. The expression of *rmf* was found to be under growth phase-dependent control and to be regulated at the transcription level. Moreover, the *rmf* expression is inversely correlated with growth rate.

The nucleotide sequences, TTGACG (−35) and TATTGC (−10), of the promoter region deduced from the position of the transcription start site deviate significantly from the consensus sequences recognized by RNA polymerase containing the major σ^{70} factor, especially in lacking the almost invariant T at the downstream end of the −10 hexamer (Harley and Reynolds, 1987; Helmann and Chamberlin, 1988). In fact, *rmf* was not transcribed *in vitro* by purified RNA polymerase containing σ^{70} (Ozaki,M., unpublished). The sequences show an even poorer match to the consensus promoters for holoenzymes carrying known minor σ factors (Helmann and Chamberlin, 1988).

Recently, stationary phase-specific genes have been identified (for references see Bohannon *et al.*, 1991; Siegele and Kolter, 1992). Among them, the genes for microcin B17 production, the morpho-gene *bolA* and the cell division gene *ftsQ* have been shown to exhibit an inverse relationship with growth rate (Connell *et al.*, 1987; Aldea *et al.*, 1990). The promoters for these genes as well as *katE* and *xthA*, encoding catalase HPII and exonuclease III, respectively, have some common sequence features. An alternative principal σ factor encoded by *katF* (*rpoS*) is considered to be directly or indirectly involved in transcription of these genes, except for the microcin B17 operon (Bohannon *et al.*, 1991; Lange and Hengge-Aronis, 1991a). The region upstream of the transcription start site of *rmf*, however, does not contain the same sequence features, and *rmf* is expressed in the normal way in a strain lacking *katF*. Thus, we conclude that the *katF* product does not have any significant role in the expression of *rmf*.

Disruption of *rmf* resulted in two detectable phenotypes in stationary phase cells; loss of viability and inability to form ribosome dimers. Since our preliminary experiments indicated that exponentially growing cells at very low rates contain ribosome dimers depending on the presence of *rmf* (data not shown), it is possible that ribosome dimer formation is correlated with the expression of *rmf* not only during growth transition to stationary phase but also under other growth conditions. However, detailed quantitative analysis to prove this correlation remains to be done.

Loss of *katF* accelerates cell death during stationary phase due to the failure of induction of the genes required to survive in this phase (Lange and Hengge-Aronis, 1991b). *katF* is, however, not essential for the expression of *rmf*. Thus, RMF is probably involved in another pathway for the maintenance of viability, or it is necessary for the action of the *katF* product. A small number of other genes have been identified that are required for viability during stationary phase (Tormo *et al.*, 1990), but the functions of these genes and their relationship with *rmf* or *katF* are not known.

The pattern of growth phase- and growth rate-dependent expression of *rmf* is in sharp contrast to those of the genes encoding ribosomal components and ribosome-associated factors involved in protein synthesis. These genes are coordinately controlled to supply appropriate amounts of translational apparatus to maintain the level of protein synthesis under most growth conditions, and their expression increases as growth rate increases. Ribosome synthesis is controlled by feedback regulation; transcriptional regulation of rRNA synthesis and translational regulation of ribosomal protein synthesis (for reviews see Nomura *et al.*, 1984; Lindahl and Zengel, 1986; Jinks-Robertson and Nomura, 1987). The synthesis of translational initiation and elongation factors also increases as growth rate increases (Pedersen *et al.*, 1978; Howe and Hershey, 1983; Grunberg-

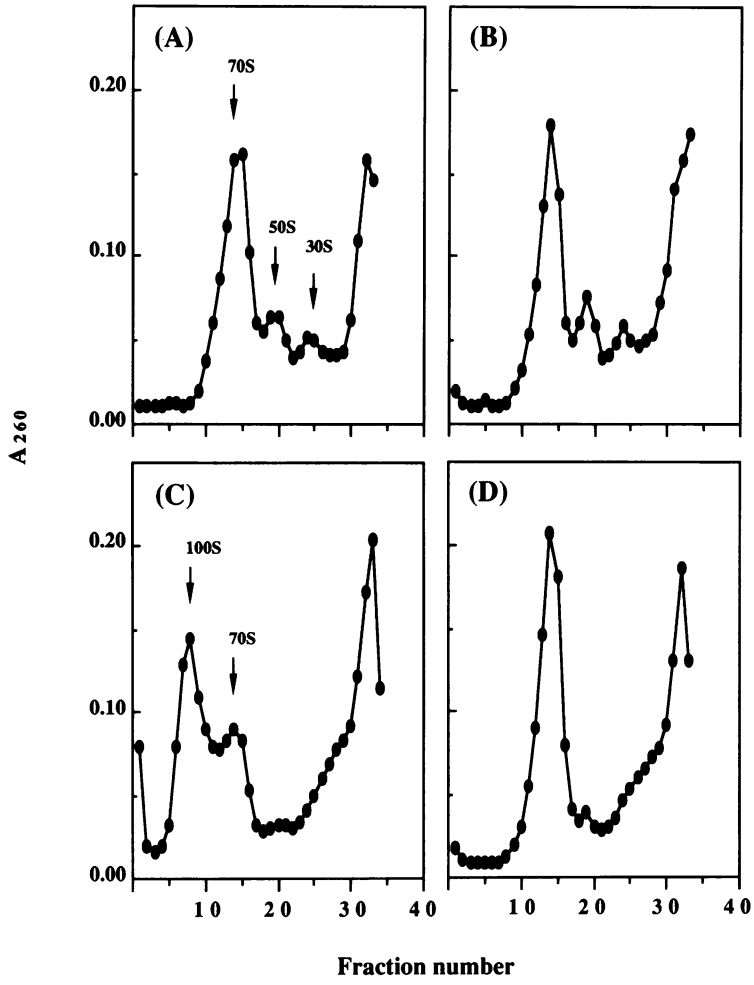

Fig. 6. Sedimentation analysis of ribosomes. Extracts of W3110 (**A** and **C**) and HMY15 (**B** and **D**) were prepared from exponential phase cultures (A and B) or stationary phase cultures (C and D) (Wada *et al.*, 1990) (the times of sampling are indicated by arrows in Figure 5) and sedimented through a 5 ml gradient of 5–20% sucrose in 20 mM Tris–HCl (pH 7.6), 15 mM magnesium acetate, 100 mM ammonium acetate in a Hitachi RPS50-2 rotor at 40 000 r.p.m. for 80 min at 4°C.

Table I. Bacterial strains

Strains	Relevant characteristics	Reference or source
W3110	*lac*	Hill and Harnish (1981)
HMY13	W3110 (λ*rmf–lacZ i*21)	This study
HMY14	W3110 *katF*::Tn*10* (λ*rmf–lacZ i*21)	This study
HMY15	W3110 *rmf*::Cm^r	This study

Manago, 1987) and some of them are under feedback regulation (for a review see Grunberg-Manago, 1987). Ribosomes are overproduced at low growth rates (Koch, 1970, 1971; Jinks-Robertson and Nomura, 1987). It is possible that overproduced ribosomes stay as dimers by association with RMF. Further studies on the function of RMF and the activity of ribosome dimers in translation will open a way to understanding the strategies of bacterial cells for survival during stationary phase.

Materials and methods

Bacterial strains and media

The strains used in this study are listed in Table I. LB, M9 and E media were as described in Davis *et al.* (1980). M9A medium is M9 containing 0.2% casamino acids, and EP medium is E containing 2% peptone. Glucose and glycerol were added at 0.4%.

Cloning and sequencing of rmf

A 4 kb *Pst*I fragment containing *rmf* was cloned from λ222 (Kohara *et al.*, 1987) into pACYC177, resulting in pT6. The nucleotide sequence of *rmf* on pT6 was determined using primers 1 and 2 (see Figure 1) by the dideoxy chain termination method (Sanger *et al.*, 1977). The *rmf* sequence was also determined using a PCR product from the W3110 genomic DNA. PCR was carried out according to Carothers *et al.* (1989).

Disruption of rmf

The *Pst*I fragment described above was recloned into M13mp18 and the nucleotide 23 of the *rmf* coding region was mutagenized from G to T to generate a *Bgl*II site according to Kramer and Frits (1987). The *Pst*I fragment containing the mutagenized *rmf* was cloned into pACYC177 to make pT3005. To disrupt *rmf*, the Cm^r gene on a *Bam*HI fragment from pHP45Ω-Cm (Fellay *et al.*, 1987) was inserted into the *Bgl*II site of pT3005

to make pT3101. A *Pst*I fragment containing *rmf*::Cm^r was isolated from pT3101 and transformed into a *recBC* strain, JC7623 (Horii and Clark, 1973). The disruption of *rmf* on the chromosomes of Cm^r transformants was confirmed by Southern hybridization. *rmf*::Cm^r was then introduced into strain W3110 by P1 transduction to generate strain HMY15.

Construction of an rmf–lacZ fusion

Both a 1.4 kbp *Hin*cII fragment of pT3005 containing the mutagenized *rmf* and the *Bam*HI fragment of pHP45Ω-Cm containing the Cm^r gene were cloned into the *Hin*cII and *Bam*HI sites of pUC18, respectively, to give pT2003. A *Bam*HI fragment carrying *lacZ* with a deletion of the first eight codons was isolated from pMC1871 (Pharmacia) and inserted into the *Bgl*II site of pT2003. The resulting plasmid, pT2101, carried an in-frame translational fusion, *rmf–lacZ*. A *Pst*I–*Kpn*I fragment containing *rmf–lacZ* and the Cm^r gene from pT2101 was treated with T4 DNA polymerase (Takara) and ligated to a left arm derived from a *Sma*I digest of λ and to a right arm derived from a *Pma*CI digest of λ*i*21*nin*5. The resulting phage, λ*rmf–lacZ i*21, was integrated into the W3110 or HMY12 chromosomes to give strains HMY13 and HMY14, respectively. Strain HMY12 was constructed by P1 transduction of *katF*::Tn*10* from UM122 (Loewen and Triggs, 1984) to W3110.

RNA analysis

Methods for isolation of total RNA and for Northern hybridization after denaturation of RNA with glyoxal were as described in Miller (1972) and in Sambrook *et al.* (1989), respectively. The *rmf* probe was a ^{32}P-labeled antisense RNA synthesized with SP6 RNA polymerase from *Bam*HI-digested pRMF11, which had been constructed by cloning of an *Acc*II–*Hin*cII fragment of λ222 containing *rmf* into the *Hin*cII site of pSPT18 (Pharmacia) such that *rmf* is counter-transcribed from the SP6 promoter. In primer extension analysis, primers were 5′ end-labeled with [γ-^{32}P]ATP and extended with Moloney murine leukemia virus reverse transcriptase (Life Technologies) as described by Domdey *et al.* (1984).

β-Galactosidase assay

The specific activity was determined as described by Miller (1972). For determination of β-galactosidase activity in exponential phase cells, overnight cultures were diluted and grown for several generations until constant levels of activity were obtained.

Acknowledgements

We thank Dr Y.Kohara for providing λ222, Dr H.Inokuchi for λ*i*21 *nin*5, Dr A.Higashitani for pH45Ω-Cm and Dr A.Nishimura for strains JC7623 and UM122. We thank Dr R.S.Hayward for critical reading of the manuscript. This work was supported by Grants-in-Aid from the Ministry of Education, Science, and Culture of Japan.

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Received on October 21, 1992